



Interstitial lung disease in systemic sclerosis: comparison of BALF lymphocyte phenotype and DLCO impairment

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Patients with scleroderma (systemic sclerosis—SSc) frequently develop an interstitial lung disease. The role of lymphocytes in fibrosing alveolitis preceding lung fibrosis has been established. The purpose of this work was to evaluate cell profiles and lymphocyte phenotypes in the bronchoalveolar lavage (BAL) fluid and to correlate them with depression in lung function tests detected by depletion of diffusing capacity (DLCO).

BAL was carried out in 25 untreated, non-smoking patients suffering from diffuse scleroderma and in 12 healthy non-smoking volunteers. For the analysis of lymphocyte sub-sets flow cytometry and monoclonal antibodies were used. The following cell sub-types were counted: T lymphocytes, B lymphocytes, helper lymphocytes, suppressor/cytotoxic lymphocytes, natural killer cells, cytotoxic T lymphocytes and activated T lymphocytes.

The total cell count was higher in the group of patients with mild and moderate impairment in DLCO. The percentage of lymphocytes was greater in patients with DLCO lower than 65% of the predicted value since neutrophilia was found in patients with severe DLCO depletion, i.e. significant when compared with healthy subjects. The proportions of suppressor/cytotoxic lymphocytes and of activated T lymphocytes were higher in patients than in controls. The statistical analysis revealed significant differences between patients with moderate and mild changes in DLCO and the healthy volunteers. A decreased helper/suppressor ratio was noticed in these patients.

We concluded that the BALF lymphocyte phenotype analysis may reflect the features of alveolitis in patients with SSc.

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Introduction

Scleroderma (systemic sclerosis—SSc) is a multisystem disorder. Fibrosis of the pulmonary parenchyma is a serious complication and has been found in about 80% of patients with SSc (1). The fibrotic process is closely linked to an inflammatory reaction known as fibrosing alveolitis (2). The role of lymphocytes in the pathogenesis of this process in a sclerotic lung has been well documented by microscopic studies (3–5). Immunosuppressive therapy may be effective (6,7) so that early diagnosis of lung involvement in SSc is very important.

We have used bronchoalveolar lavage (BAL) to analyse lung cell profiles in patients with systemic sclerosis and in healthy non-smoking volunteers. The value of this method in patients suffering from SSc has generally been recognized

(8–10). In our study BAL was performed in patients with minimal lung involvement, thereby this examination was well tolerated (9). In the BAL fluid (BALF) cells analysis, typical signs of fibrosing alveolitis such as a high total cell count, lymphocytosis and low CD4+:CD8+ cells ratio, resulting from an elevated CD8+ lymphocyte percentage, were observed in patients with various stages of lung involvement (9,11,12). The diffusing capacity for carbon monoxide (DLCO) has been found to be the most sensitive physiological parameter for the early lung changes in the course of fibrosing alveolitis (4,13,14). The aim of this part of our study was to find a correlation between DLCO depression and any possible changes in the BALF, particularly those in lymphocyte sub-populations.

Methods

SUBJECTS

The group of patients consisting of 25 subjects (23 women and two men) met the American Rheumatism Association preliminary criteria for the diagnosis of scleroderma (15).

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TABLE 1. Results of lung function tests in patients with systemic sclerosis

	Number	Per cent predicted (mean)
VC <70% N	6/25 (24%)	85 ± 22
FEV ₁ <70% N	6/25 (24%)	79 ± 18
FEV ₁ %VC <70% N	3/25 (12%)	80 ± 11
VC <70% N and FEV ₁ <70% N	3/25 (12%)	
TGV >120% N	3/25 (12%)	104 ± 17
D _L CO		61 ± 16
D _L CO <50% N	7/25 (28%)	41 ± 7.5
D _L CO 50–65% N	10/25 (40%)	60 ± 3.5
D _L CO >65% N	8/25 (32%)	77 ± 13
D _L CO <50% and VC <70%	4/25 (16%)	

VC, vital capacity; FEV₁, forced expiratory volume; TGV, thoracic gas volume; D_LCO, diffusing capacity for carbon monoxide; N, normal values for sex, age and height.

The diagnosis was established at the Dermatology Department, Warsaw Medical School. All patients presented clinical symptoms of diffuse scleroderma. In 15 (60%) of them autoantibodies specific to scleroderma TOPO I were positive. The patients ranged in age from 28 to 74 years (mean 48 years). The mean duration of the disease from the first signs of Raynaud's phenomenon was 8 years (from 1 to 17 years). Smokers were excluded because abnormalities in the lavage fluid have been associated with cigarette smoke inhalation (16). The patients did not receive immunosuppressive therapy for at least 2 months prior to and at the time of lavage. All SSC patients underwent clinical evaluation, chest radiography and pulmonary function tests before the BAL examination at the Department of Pneumology. Vital capacity (VC) and forced expiratory volume (FEV₁) were determined using a Jaeger spirometer. Lung diffusion capacity was measured by steady-state method (D_LCOSS). In short: for 2 min patients breathe a mixture containing 0.1–0.2% CO; for the next 2 min every 30 s CO concentration in exhaled air and in end-tidal 100–200 ml was measured. The minute ventilation (MV) and CO concentration in inhaled, exhaled and end-tidal mixture were calculated. These values enabled us to calculate D_LCO (D_LCO = V/Pa where V indicates CO volume diffused from alveolar to capillary blood and Pa indicates alveolar CO partial pressure). The predicted values for each subject based on sex, age and height were obtained from standard tables (17). The patients were divided in relation to the D_LCO impairment as mild, moderate and severe if percentages predicted were higher than 65%, 50–65% and less than 50% respectively [(18) modified]. The results of pulmonary function tests are summarized in Table 1.

In addition to the group of patients we also evaluated 12 normal healthy non-smoking volunteers: six women and six men, aged 23–66 years (mean age 35 years).

All patients and normal controls were instructed about the risk of the study and gave their informed consent. The study was approved by the Ethics Committee of Warsaw Medical School.

METHODS

Bronchoalveolar lavage

The patients and controls underwent BAL after pre-medication with atropine and under lidocaine upper respiratory tract anaesthesia. After tracheobronchial tree examination the bronchofibroscopy was wedged in a sub-segment of the right middle lobe. A 200-ml sterile saline lavage was performed by sequential instillation of 50-ml aliquots, which were subsequently immediately removed (19).

Analysis of BALF cells

The BAL fluid was filtered through two layers of surgical gauze, and the volume of the removed fluid was measured. The volume of the recovery fluid larger than 50% was regarded as being of diagnostic value. The fluid was centrifuged at 4°C at 300 g for 10 min. Cell viability was determined by trypan blue dye exclusion. The total cell count and a differential count was performed on smears stained with the May-Grunwald-Giemsa (MGG) (300 cells from each sample were counted) (19).

For flow cytometry, the samples of cell suspension (mean 10⁶ cells ml⁻¹) were incubated with a mixture of two appropriate antibodies at 4°C for 30 min in the dark, 6 µl of mixture of antibodies being added to 50 µl of the cell suspension. Then the cells were washed twice with PBS and resuspended in PBS containing 0.5% formaldehyde. For double colour analysis the antibodies were conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE). The antibodies recognizing the following antigens were used in pairs: CD45 (FITC)/CD14 (PE), CD3 (FITC)/CD19 (PE), CD4 (FITC)/CD8 (PE), CD3 (FITC)/HLADR (PE), CD3 (FITC)/CD56 (PE) and CD16 (PE) (all from Becton-Dickinson) and CD25 (FITC)/CD3 (PE) (from Dako). Antibodies anti-CD16 and CD56 conjugated with phycoerythrin were used together. In each analysis, cells stained by FITC- and PE-conjugated isotype mouse-IgG

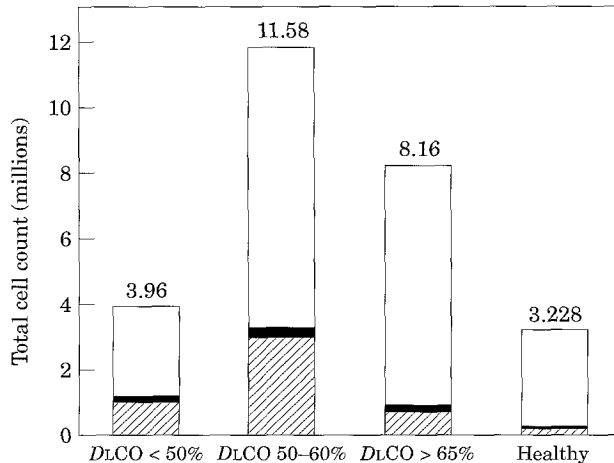


FIG. 1. Differences in the total cell count in the BALF from patients with severe, moderate and mild impairment in diffusing capacity (DLCO) in SSc patients and healthy subjects. (□), Alveolar macrophages; (■), neutrophils; (▨), lymphocytes.

were employed as negative control. Flow cytometric analyses were processed by Simul SET software in a FAC Strac cytometer (Becton-Dickinson). Eight to 10×10^4 cells from each sample were counted and lymphocyte population were selected based on side scatter and expression of CD45 antigen. The data were analysed using a PC Lysis programme (Becton-Dickinson). For data comparison the CD4+:CD8+, CD4+:CD3+ and CD8+:CD3+ ratios were counted (20).

Statistical analysis

A non-parametric analysis was used to make group comparisons of data since BAL cellularity was not distributed normally. Median and 0.25–0.75 percentile values were used. The Mann–Whitney *U*-test was applied for the comparisons of the groups (21), *P*-value <0.05 being regarded as significant. The relationship between the proportion of

lymphocyte subtypes and the DLCO was tested by the Spearman correlation test.

Results

LAVAGE FLUID AND CELLULARITY

The mean recovery volume of the fluid was 130 ml. The median of the total cell count in the BALF from patients was 7.6×10^6 , which was higher than that in healthy persons [3.22×10^6 ($P < 0.05$)]. Patients with moderate and mild DLCO changes had a greater total cell count ($P < 0.01$ in comparison with healthy persons) which is shown in Fig. 1. The proportion of lymphocytes and neutrophils in the lavage from patients differed significantly from the lavage from normal persons (Table 2). We have found an increased proportion of lymphocytes in the BALF in patients with severe and moderate impairment of DLCO than in the control group ($P < 0.05$). The difference found in the percentage of neutrophils was significant in patients with severe DLCO depletion when compared with healthy persons ($P < 0.05$). As shown in Fig. 1 total lymphocytes count was also higher in patients with depletion of DLCO <65%. The total count of the alveolar macrophages was higher in the group of patients, but the analysis of these cells was not the subject of the study.

LYMPHOCYTE PHENOTYPING

The median percentages of lymphocytes sub-sets in the BALF from patients with different changes in DLCO and in healthy persons are summarized in Table 3. The median proportion of T lymphocytes CD3+ in the BALF in patients and controls did not differ significantly. The number of B lymphocytes CD19+ in the BALF from patients was higher but the difference with the control group was not significant.

Patients suffering from SSc had a greater proportion of cytotoxic/suppressor CD8+ lymphocytes and an increased CD8+ to CD3+ lymphocyte ratio. The difference was

TABLE 2. Differential cell profiles obtained with BAL in healthy persons and in patients suffering from SSc (smears stained with May–Grunwald–Giemsa)

Cells	Healthy subjects <i>n</i> =12	Patients <i>n</i> =25		
		DLCO <50% <i>n</i> =7	DLCO 50–65% <i>n</i> =10	DLCO >65% <i>n</i> =8
Lymphocytes (%)	9 (8–11.5)	22* (14–30)	25.5* (8–30)	10 (7.5–21.5)
Macrophages (%)	89.5 (84–91)	70* (65–79)	70* (58–79)	84.5 (73–90.5)
Neutrophils (%)	1.5 (0.5–2)	4* (2–6)	2 (0–5)	2 (0.5–3)

Median values and lower–upper quartile in brackets.

* $P < 0.05$ Mann–Whitney test vs control group.

TABLE 3. Results of BALF lymphocyte phenotyping in patients with severe (<50% predicted), moderate (50–65%) and mild (>65%) impairment in the diffusing capacity for carbon monoxide (DLCO) and in healthy subjects

Lymphocyte sub-set	Healthy (n=12)	Patients (n=25)		
		DLCO <50% I (n=7)	DLCO 50–65% II (n=10)	DLCO >65% III (n=8)
CD3+	68.0	62.0	70.0	65.0
(T lymphocytes)	(49.5–77.5)	(50.0–72.0)	(61.0–84.0)	(60.0–69.5)
CD19+	1.0	1.0	3.0	1.0
(B lymphocytes)	(1.0–1.5)	(0–3.0)	(1.0–6.0)	(0–2.0)
CD4+	41.5	26.0	25.0	28.5
(helper)	(25.0–49.0)	(17.0–37.0)	(22.0–31.5)	(18.0–33.0)
CD8+	22.5	26.0	38.5*	37.5
(cytotoxic/suppressor)	(4.0–27.0)	(14.0–29.0)	(26.0–43.0)	(19.5–42.0)
CD3 – /CD16+CD56+	4.0	2.0*	5.0	5.0
(natural killer)	(3.0–5.5)	(1.0–2.0)	(3.0–5.5)	(2.5–7.0)
CD3+/HLADR+	10.5	13.0	16.0	11.0
(activated lymphocytes)	(5.0–15.5)	(5.0–14.5)	(8.0–18.0)	(8.0–15.0)
CD3+/CD16+CD56+	3.0	4.0	4.0	4.5
(cytotoxic lymphocytes)	(2.0–5.0)	(3.0–6.0)	(3.0–5.0)	(2.0–6.5)
CD25+/CD3+	2.0	4.0*	3.0	4.5*
(activated lymphocytes)	(0–3.0)	(2.0–4.0)	(1.0–4.0)	(2.0–6.5)

* $P<0.05$ in Mann–Whitney *U*-test.

TABLE 4. Ratios of lymphocyte sub-types in the three groups of patients and in healthy volunteers

Ratio of lymphocyte sub-types	Healthy (n=12)	Patients (n=25)		
		DLCO <50% I (n=7)	DLCO 50–65% II (n=10)	DLCO >65% III (n=8)
CD4+:CD3+	0.63	0.55	0.42*	0.39*
	(0.58–0.72)	(0.42–0.64)	(0.35–0.62)	(0.32–0.48)
CD8+:CD3+	0.36	0.42	0.59*	0.54*
	(0.25–0.41)	(0.35–0.57)	(0.32–0.61)	(0.44–0.58)
CD4+:CD8+	1.68	1.53	0.68*	0.70*
	(1.21–2.82)	(0.89–2.00)	(0.51–0.82)	(0.59–1.10)

* $P<0.05$ in Mann–Whitney *U*-test.

significant in the group with moderate DLCO depletion when compared with healthy persons for percentage of CD8+ lymphocytes. When the CD8+:CD3+ (T lymphocytes) ratio was compared differences were found for groups II and III (Table 4). The proportion of CD4+ lymphocytes was lower in BALF from patients, but only the CD4+:CD3+ ratios differed significantly when BALF from healthy subjects were compared with BALF from patients with moderate and mild DLCO impairment (Table 4). The CD4+:CD8+ ratios were decreased in BALF from patients in the groups with moderate and mild DLCO impairment when compared with the controls, the differences being significant ($P<0.05$). There were higher proportions of activated T lymphocytes with

HLA DR as well as CD25 antigen expressions in the BALF from patients and they were significant for the CD25+:CD3+ lymphocyte percentage between the group with mild DLCO depletion and the controls. Interestingly, the proportion of NK cells was significantly lower in the BALF from patients with severe DLCO depletion. For cytotoxic CD16+/CD56+ T lymphocytes no significant differences were found, though there were higher proportions of these cells in patients with moderate and mild changes in DLCO.

The proportion of lymphocyte sub-types did not correlate with the depletion in either VC or FEV₁ nor in both. There was no significant correlation between the lymphocyte phenotype and the value of DLCO.

Discussion

Lung fibrosis is a common and most serious complication in more than 50% of patients with scleroderma. It has been well documented that single-site lavage is an acceptable method to assess pulmonary inflammation. The aim of our study was to determine if the analysis of cell profiles and lymphocyte phenotyping in the BALF may be useful in the detection of the early stage of the lung disease. We have performed BAL in patients with different durations of the disease. Previous studies have observed that duration of SSc symptoms does not correlate with severity of internal organ involvement (12,22). In our previous study we did not find any correlation between BALF findings and the duration of SSc symptoms (9). Most of our patients did not reveal typical features of advanced lung fibrosis apart from DLCO depletion. We have found typical clinical signs of lung fibrosis in two patients with severe DLCO depletion. There were typical radiological features in five patients. Only six patients revealed VC depletion and in three subjects this was caused by obturation of the bronchial tree. Bronchial obturation has been found previously to be a feature of lung involvement in the course of scleroderma by Kane (23) and Owens (24). The next four patients had reduction in both VC and DLCO. The diffusing capacity ranged from 32% to 80% of the predicted value in patients. In most patients (76%) it was an isolated change. In Owens's study, isolated DLCO reduction was observed in 26 out of 80 subjects (24), and in Kane's it was found in 31% of patients with diffuse SSc (23). There are only few studies in which BALF changes in the early stages of alveolitis have been investigated (12,22,25). Since the depletion of DLCO to about 50% of the predicted value results from the fibrosis of the alveolar wall, active alveolitis with lesser DLCO impairment may reflect a reversible process in the alveolar lumen (25–27). In his recent study Wells pointed out the role of measuring DLCO (28). We have compared the results of BALF analysis in three groups of patients with three degrees of DLCO reduction and with healthy volunteers, smokers and infected persons being excluded. The BAL procedures, mean volumes of the recovered fluid and cell viability were comparable in all subjects and controls.

In the BALF analysis, the main change was an increase in the BALF total cell count in patients as compared with controls, especially in patients with moderate impairment of DLCO. This is a common feature of alveolitis and in patients with collagen-vascular disease it is regarded as a sign of active lung disease. In this study, the total cell count normalized in patients with DLCO <50% and restrictive changes. As also noted by Owens, the recent stage of lung fibrosis is characterized by elevated cellularity (12). The most important finding in fibrosing alveolitis may be the greater proportion of lymphocytes and/or neutrophils (7,22,25,29). Data differ from study to study. Interestingly, we noticed the greatest proportion of lymphocytes in SSc patients with moderate and severe impairment of DLCO when compared with healthy subjects. The total number of these cells was also higher in these two groups. In the whole group of patients we noticed a higher percentage of neutrophils: from 1 to 24% and there were no significant

differences between these three groups of patients. The mean proportion of neutrophils was higher and differed significantly between patients with severe DLCO reduction and controls. Wells has found neutrophilia accompanying extensive changes in lung CT (30). In our observation, lymphocytosis as well as neutrophilia are the features of lung involvement with advanced changes in DLCO.

The role of suppressor/cytotoxic lymphocytes with CD8 antigen expression in the pathogenesis of fibrosing alveolitis in scleroderma has been well documented (9,31,32). In our studies, the increased proportion of these cells was appreciable in patients with moderate and mild changes in the diffusing capacity as well in the total group of patients as compared with healthy persons. Owens (12) and Frigieri (11) have observed a greater proportion of CD8+ cells in patients with SSc. Trentin has provided an explanation for the participation of CD8+ lymphocytes in alveolitis in hypersensitivity pneumonitis (HP) (33). His suggestion was that the increased number of CD8+ cells resulting from long-term activation and stimulation by an unknown agent may be real in the pathogenesis of alveolitis in scleroderma. The CD8+ cells lung compartmentalization was observed (34), because the analysis of blood lymphocytes led to different results: elevated percentage of CD4+ cells with a decreased proportion of CD8+ lymphocytes (35).

In this study we have found a greater proportion of cytotoxic lymphocytes with expression CD3, CD16 and CD56 antigens in some patients with minimal changes in DLCO. It is known that the proportion of cytotoxic lymphocytes is higher than in the blood (32) but the role of cytotoxicity requires further investigation (34).

One of the most important features of alveolitis is a marked participation of activated lymphocytes (3,36). The proportion of the HLA DR+ lymphocytes T in the BALF in patients did not differ significantly when compared with the control group. However, in patients with moderate DLCO depletion the mean proportion of HLA DR+ T lymphocytes was highest. The proportion of CD25+ lymphocytes in the lung is generally low (32,37). In our study, there was a relationship between percentage of lymphocytes CD25+/CD3+ and the value of the diffusing capacity (DLCO): in patients with a mild degree of DLCO depletion the percentage of these cells was higher. Our observations correlate with Haslam's findings in lung biopsies: that a small number of lymphocytes with IL-2 receptors were identified in the sclerotic lungs (3).

In conclusion, a greater total number of cells, a high proportion of CD8+ lymphocytes with a decreased CD4+:CD8+ ratio and a high proportion of activated T lymphocytes CD3+/CD25+ in the BALF may be observed in patients with SSc without clinical signs of lung fibrosis and with isolated mild or moderate DLCO impairment.

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